Dominance of *Lactobacillus acidophilus* in the Facultative Jejunal *Lactobacillus* Microbiota of Fistulated Beagles

Yurui Tang, Titta J. K. Manninen, and Per E. J. Saris

Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland

Lactobacilli were isolated from jejunal chyme from five fistulated beagles. Cultivable lactobacilli varied from 10^4 to 10^8 CFU/ml. Seventy-four isolates were identified by partial 16S rRNA gene sequencing and differentiated by repetitive element PCR (Rep-PCR), *Lactobacillus acidophilus* was dominant, and nearly 80% of 54 isolates shared the same DNA fingerprint pattern.

Probiotic bacteria are eaten to restore the balance of the intestinal microbiota ([7], [14]) and have other health-promoting potential, such as modifying gut microflora, enhancing nutrient digestibility, strengthening the gut mucosal barrier, regulating the immune system, and reducing gastrointestinal (GI) disorders ([15], [18], [19]). In addition, they have recently reported to affect brain function ([6]). Among probiotic lactic acid bacteria (LAB), *Lactobacillus* species are the most utilized, and some of them have been studied to improve the health of dogs ([3], [22]). However, knowledge of canine intestinal microbiota is still limited, and most commercial canine probiotic products are confined to bacteria of human origin ([23]). Host specificity is an important criterion for selection of a probiotic ([8], [21]). Therefore, probiotic bacteria with greater specificity for dogs are needed.

Due to sampling difficulty, the canine intestinal LAB microbiota has been studied mainly from feces ([9], [11]). However, it is argued that fecal samples cannot represent the microbiota present in the upper gut since they are different niches and provide specific habitats for different microbiota ([17]). Therefore, studies based on GI samples are needed to reflect the actual community in GI tract. A fistulated dog model for obtaining fresh intestinal samples from healthy dogs without disturbing the physiology of the gut has been introduced ([12], [16]). One aim of this study was to investigate the canine facultative jejunal *Lactobacillus* microbiota of fistulated beagles since it is poorly known, and oxygen-tolerant lactobacilli are more feasible than anaerobes for industrial production of probiotics.

Five permanently fistulated beagles (one female and four castrated males, from 4 to 8 years of age) from the experimental animal colony unit at the University of Helsinki were selected for the study. Through an operation, all dogs had permanent jejunum nipple valve fistulas inserted into the proximal jejunum, 60 cm distally from the pylorus. The operations were performed 3 to 6 years before this study took place according to the method described earlier ([25]). The dogs had been used only for sampling of jejunal chyme and had never been under antimicrobial treatment. Jejunal chyme was collected via the nipple valve fistula by using a sterile plastic tube. The dogs were fed with dry commercial balanced dog food (protein, 23%; fat, 13%; fiber, 2.5%; ashes, 8%; calcium, 16 g/kg; phosphorus, 12 g/kg; moisture, 8%). The study was approved by the University of Helsinki Ethics Committee. One jejunal chyme sample from each dog was taken 2 h after the morning feeding and then plated on triplicate nutrient agar (NA) plates (Difco, Becton, Dickinson, Sparks, MD) and *Lactobacillus*
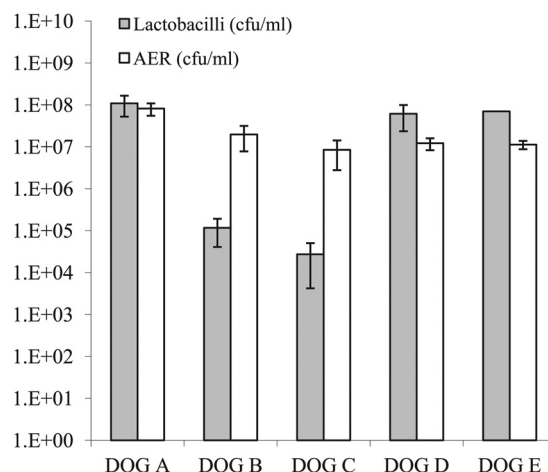
FIG 1 Total facultative bacterial counts and lactobacillus viable counts (CFU/ml) detected in the jejunal chyme from five beagles. Data are presented as mean values ± standard deviations (SD) ($n = 3$), except for dog E, for whom the viable count is given as the mean value of a duplicate plate. AER, aerobes.

selection (LBS) plates (BBL, Becton, Dickinson Microbiology System, Cockeysville, MD) without acetic acid (mLBS) ([4]), with serial dilution of 10^{-2} to 10^{-10} within 3 h of sampling. The samples then were cultivated aerobically for 48 h at 37°C.

From each dog's jejunal chyme mLBS plates, approximately 20 colonies were picked randomly. Colonies were grown in 2 ml of mLBS broth, and chromosomal DNA was isolated according to the method described by Manninen et al. ([16]). A total of 74 isolates from five dogs' jejunal chyme were identified by partial 16S rRNA gene sequencing and then compared against the National Center for Biotechnology Industry (NCBI) BLAST Library (http://www.ncbi.nlm.nih.gov). An identity of higher than 98% was used as the criterion for species identification of the isolates.

Strain-level typing of 54 jejunal *Lactobacillus acidophilus* iso-

TABLE 1 *Lactobacillus* species isolated from canine jejunal chyme

Lactobacillus species	No. (%) of isolates from dog[a]:				
	A (n = 17)	B (n = 17)	C (n = 11)	D (n = 14)	E (n = 15)
L. acidophilus	15 (88.2)	12 (70.6)	2 (18.2)	14 (100)	15 (100)
L. murinus	1 (5.9)	4 (23.5)	7 (63.6)		
L. reuteri		1 (5.9)	2 (18.2)		
L. johnsonii	1 (5.9)				

[a] Isolates were obtained from jejunal chyme plated on mLBS and grown aerobically. *n*, number of isolates.

lates out of 58 sequenced *L. acidophilus* isolates was done by repetitive element PCR (Rep-PCR) using the (GTG)$_5$ primer (24) and chromosomal DNA as a template. Applicability of this method for identification of *Lactobacillus* at the species, subspecies, and strain levels was demonstrated by Gevers et al. (10). In addition to canine-derived *L. acidophilus* strains, non-canine-origin *L. acidophilus* strains were studied to show the differentiation capacity of Rep-PCR. These controls included *L. acidophilus* strains LAB48, a chicken crop isolate (1), HAMBI80 (from the Culture Collection at the Department of Microbiology, University of Helsinki), HAMBI1448, 74-2 (Danisco, Ltd.), and ATCC 4356 (American Type Culture Collection).

In our study, we investigated aerobically cultivable lactobacilli isolated from the jejunal chyme from five fistulated beagles. The

jejunal fistulated dog model was suggested as a promising method to investigate the small intestinal microflora without significant alteration of the intestinal motility and microflora (12). Besides, the nipple valve enables investigations on small intestinal samples with higher frequency than other techniques requiring endoscopy or laparotomy. Janna et al. (12) have investigated the influence of the fistula on intestinal motility by feeding dogs with barium-impregnated polyethylene spheres (BIPS). The mean orocolic transit percentages were found to be 93% before surgery and 83% after surgery, which was considered to represent no notable changes in gastrointestinal motility.

It was found that the jejunal chyme specimens from five dogs (A, B, C, D, and E) contained similar amounts of total facultative bacterial counts on NA plates, consisting of 3×10^7 CFU/ml on average. With selection for lactobacilli, variation in bacterial counts was observed (Fig. 1). While jejunal chyme from dogs A, D, and E contained 8×10^7 CFU/ml of aerobically grown lactobacilli, the other two dogs had lower counts (7×10^4 CFU/ml). In three dogs out of five, the aerobically grown *Lactobacillus* microbiota was found to dominate the facultative microbiota in jejunal chyme (Fig. 1).

Mentula et al. (17) found cultivable lactobacilli from jejunal chyme of the canine GI tract in small quantities: only 30 CFU/g of lactobacilli were found in total of 27 samples. However, we found large quantities (10^4 to 10^8 CFU/ml) of *Lactobacillus*
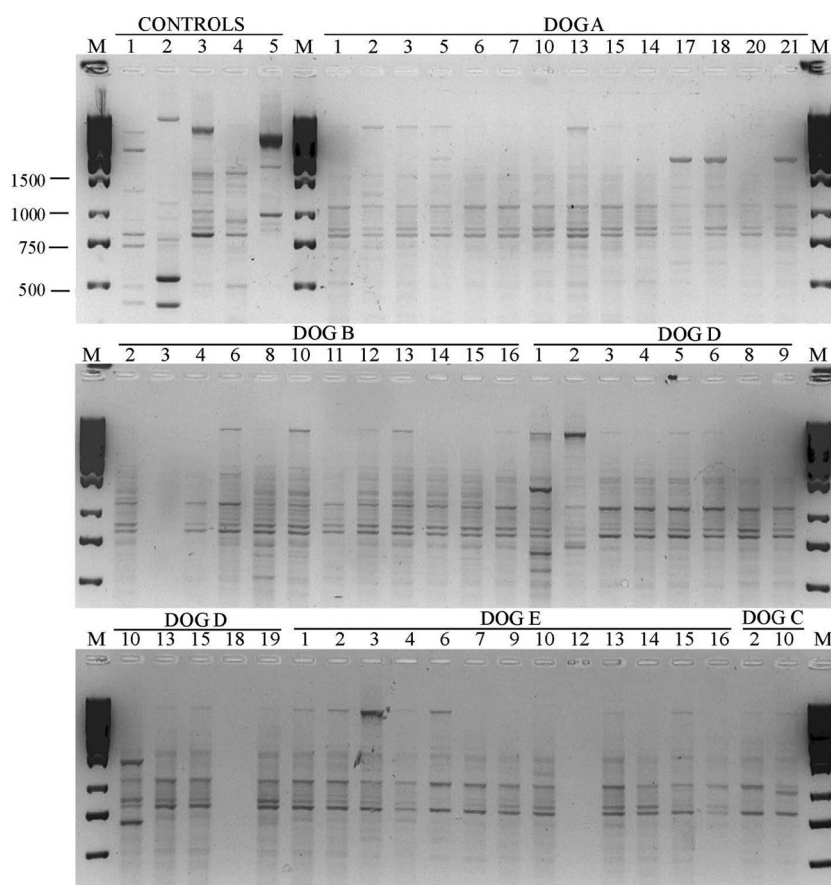
FIG 2 Rep-PCR of *L. acidophilus* strains isolated from canine jejunal chyme. Lane 1, *L. acidophilus* LAB48; lane 2, *L. acidophilus* HAMBI80; lane 3, *L. acidophilus* HAMBI1448; lane 4, *L. acidophilus* 74-2; lane 5, *L. acidophilus* ATCC 4356; lane M, 1-kbp DNA ladder. Profiles of A5, A18, B2, B8, D1, D10, and E3 were clearly different from the fingerprint pattern of the majority (for a typical representative, see A13).

strains in all of our jejunal chyme samples. One reason for this difference could arise from sample treatment. We plated fresh jejunal chyme samples within 3 h, whereas Mentula et al. froze the samples without adding cryoprotectants before plating. Freezing can inactivate bacteria (2) and may account for the reported low prevalence of cultivable lactobacilli in the jejunal chyme. Another explanation for the few isolated lactobacilli may come from the usage of MRS plates, which are not selective for lactobacilli. Staphylococci, streptococci, and enterococci also grow on MRS plates (13), and Mentula et al. reported these bacteria to be present in the jejunal chyme in amounts of 10^3 CFU/g. It is likely that these bacteria yielded more colonies on the MRS plates than the lactobacilli that survived the freezing. We used fresh jejunal chyme and plating on mLBS plates, which are selective for lactobacilli, and found that lactobacilli are present in large numbers in the jejunal chyme of canine. The mLBS plates are less selective than LBS plates due to lack of acetic acid addition, but this reduced selectivity we showed earlier was needed for isolation of lactobacilli from canine feces (5). Rinkinen et al. also plated fresh jejunal chyme from fistulated dogs, but on MRS plates (20). The amounts of bacteria in these chyme samples ranged from 10^5 to 10^6, and species determination was done based on partial 16S RNA gene sequence comparisons. Twelve *Streptococcus alactolyticus* strains, six *Lactobacillus murinus* strains, and four *Lactobacillus reuteri* strains were identified. Clearly, this result is in accordance with ours and suggests that plating of fresh jejunal chyme samples enables isolation of lactobacilli (even on MRS plates) and those lactobacilli are present in large numbers in the jejunum.

A total of 74 isolates from mLBS plates were clustered into four species. *L. acidophilus* was dominant in four dogs (Table 1), and the isolates from two of the dogs were confined to *L. acidophilus* strains. Instead of *L. acidophilus*, *L. murinus* (63.6%) dominated in the jejunal chyme of the fifth dog and was also detected in two other dogs. *L. reuteri* was found in two dogs, while *L. johnsonii* was detected only in one dog. The results indicated that the facultative jejunal lactobacilli consist of limited number of species, and these few species may dominate others. Previous results studying the microbiota of the intestine of euthanized dogs using 16S RNA gene analysis revealed similar results that lactobacilli from canine small intestine are limited to only few species, such as *L. animalis*, *L. aviarius*, *L. johnsonii*, *L. murinus*, and *L. reuteri* (20).

The variation of strain composition of isolated *L. acidophilus* strains was analyzed by Rep-PCR. By using the $(GTG)_5$-primer pair, Rep-PCR generated fragment profiles from 51 out of 58 identified jejunal *L. acidophilus* isolates (Fig. 2). The results showed that none of the strains isolated in this study were identical to any of the control *L. acidophilus* strains. Eight isolates yielded clearly different profiles from the majority of the other profiles, which potentially could be grouped into one profile type. This suggests that the representative of this strain (LAB20) of *L. acidophilus* seems to be a good candidate probiotic for a dominant facultative *L. acidophilus* strain competitive in the canine jejunum. In addition, the *L. acidophilus* bacteria were present in large numbers (approximately 10^8 CFU/g) in the jejunal contents of the dogs D and E, from whom only *L. acidophilus* strains were isolated.

In conclusion, this study showed that facultative *Lactobacillus* strains were abundant in the jejunal microbiota of five fistulated beagles, of which *L. acidophilus* was dominant. A representative strain, LAB20, which had the most common fingerprint of the *L. acidophilus* isolates was selected for further study due to its competitive and abundant presence in canine small intestine.

ACKNOWLEDGMENTS

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